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specificities.

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-binding domains, modified toxin
Fvs, has the potential to generate
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9, 293 (1991).

Moller, *Proc. Natl. Acad. Sci. U.S.A.* 85,

Wallace, M. Abrahamsen, K. Fry, and C.

D. J. Chiswell, *Nature (London)* 348, 552

Benkovic, R. A. Lerner, E. D. Getzoff, and
654 (1990).

[5] Humanization of Monoclonal Antibodies

By DETLEF GÜSSOW and GERHARD SEEMANN

Since Köhler and Milstein's breakthrough in hybridoma technology,¹ monoclonal antibodies (MAbs) have become ever more important tools in all fields of biology and medicine. A large panel of diagnostic as well as therapeutic MAbs have been developed that are of potential value for *in vivo* diagnosis and therapy in humans.^{2,3} Virtually all MAbs are of rodent origin and exposure of humans to such heterologous MAbs can severely limit their use because of allergic reactions. Immunocompetent individuals may develop human anti-mouse immunoglobulin (Ig) antibodies (HAMA).^{4,5} Human MAbs would obviously eliminate such difficulties but the technology to produce them will require considerable refinement before it becomes generally applicable.⁶ However, standard molecular biology techniques can offer a way out of the dilemma. Working on the level of the immunoglobulin genes, we are now able to recombine variable-region genes of the heavy or light chains (V_H or V_L) with constant-region genes of any desired isotype, and, more importantly, we also have the choice between constant-region genes of different species.^{7,8} Shuffling the variable domains between different antibody genes is now an established technique for creating so-called chimeric antibodies where the variable domain is of murine origin and the constant region is human. These chimeric MAbs usually resemble their parental mouse MAb in specificity as well as affinity for the antigen.^{9,10} Chimeric MAbs are already more suitable for therapeutic use in humans. However, the mouse variable region still carries a host of immunogenic epitopes and it is likely that chimeric MAbs are still immu-

¹ G. Köhler and C. Milstein, *Nature (London)* 256, 495 (1975).

² J. L. Murray and M. W. Unger, *Crit. Rev. Oncol./Hematol.* 8, 227 (1988).

³ H.-H. Sedlacek, G. Schulz, A. Steinsträsser, L. Kuhlmann, A. Schwarz, L. Seidel, G. Seemann, H.-P. Kraemer, and K. Bosslet, in "Contributions to Oncology" (S. Eckhardt, J. H. Holzner and G. A. Nagel, eds.), Vol. 32. Karger, Basel, 1988.

⁴ R. A. Miller, A. R. Oseroff, P. T. Stratte, and R. Levy, *Blood* 62(5), 988 (1983).

⁵ M. J. P. G. Van Kroonenburgh and E. K. J. Pauwels, *Nucl. Med. Commun.* 9, 919 (1988).

⁶ J. E. Boyd, K. James, and D. B. L. McClelland, *Trends Biotechnol.* 2(3), 70 (1984).

⁷ V. T. Oi, S. L. Morrison, L. A. Herzenberg, and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* 80, 825 (1983).

⁸ S. L. Morrison and V. T. Oi, *Adv. Immunol.* 44, 65 (1989).

⁹ G. L. Boulianne, N. Hozumi, and M. J. Shulman, *Nature (London)* 312, 643 (1984).

¹⁰ S. L. Morrison, M. J. Johnson, L. A. Herzenberg, and V. T. Oi, *Proc. Natl. Acad. Sci. U.S.A.* 81, 6851 (1984).

nogenic as it has been shown in a mouse model system with chimeric MABs containing murine V and human constant domains.¹¹

Jones *et al.*¹² and Riechmann *et al.*¹³ have taken another more subtle approach, in order to make a more human form of a rodent MAB. Antigen-binding sites, composed of the three CDRs (complementarity determining regions) of the heavy chain and the three CDRs of the light chain, can be taken from a rodent MAB and inserted directly into the framework of a human antibody, thus transplanting only the CDRs rather than the entire variable domain of a rodent antibody. This is achieved by the use of oligonucleotides to replace the CDRs of an appropriate human immunoglobulin gene with the CDRs from a rodent MAB with a desired specificity. The CDRs or hypervariable regions are unlikely to carry any species-specific characteristics, and therefore such a "reshaped" human antibody should be indistinguishable from genuine human immunoglobulins. Although anti-idiotypic antibodies against a reshaped or "humanized" MAB can be generated *in vivo*, the occurrence of such antibodies does not seem to be a problem in therapy (H. Waldmann, personal communication, 1989).

The general applicability of the antibody-reshaping technique relies on two assumptions: (1) the antigen-binding site is fashioned by the CDRs as defined by Kabat *et al.*,¹⁴ no other parts of the variable region taking part in binding the antigen; (2) the frameworks of the variable domains serve as a scaffold to support the CDRs in a specific way that facilitates antigen binding. Subsequently it is of great importance to retain the interactions between the donor (rodent) CDRs and the acceptor (human) framework as closely as possible to the CDR-framework interactions of the original MAB.

However, the affinity of the first fully reshaped antibody, CAMPATH1, was nearly 40-fold lower compared to the original rat MAB.¹³ Close inspection of the sequences of the hypervariable loops in the human and rat antibodies, in particular at their junctions with the framework regions of the V_H domain, showed the possible origin of the flaw. A comparison of the amino acid sequence in the flanking region of CDR1 (residues 31–35, Kabat *et al.*¹⁴) showed that the original rat antibody has a phenylalanine at

¹¹ M. Brüggemann, G. Winter, H. Waldmann, and M. S. Neuberger, *J. Exp. Med.* **170**, 2153 (1989).

¹² P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter, *Nature (London)* **321**, 522 (1986).

¹³ L. Riechmann, M. Clark, H. Waldmann, and G. Winter, *Nature (London)* **332**, 323 (1988).

¹⁴ E. A. Kabat, T. T. Wu, M. Reid-Miller, H. M. Perry, and K. S. Gottesman, "Sequences of Proteins of Immunological Interest," 4th Ed., U.S. Department of Health and Human Services, US Washington, D.C., 1987.

se model system with chimeric constant domains.¹¹

have taken another more subtle form of a rodent MAb. Anti-CDRs (complementarity determined three CDRs of the light chain, grafted directly into the framework of only the CDRs rather than the heavy chain). This is achieved by the use of an appropriate human immunoglobulin MAb with a desired specificity. It is unlikely to carry any species-specific "reshaped" human antibody into human immunoglobulins. Although reshaped or "humanized" MAB of such antibodies does not seem to be a problem, personal communication,

the reshaping technique relies on the site is fashioned by the CDRs as the variable region taking part in the variable domains serve as a specific way that facilitates antigen interaction to retain the interactions of the acceptor (human) framework as the work interactions of the original

reshaped antibody, CAMPATH1, the original rat MAb.¹³ Close invariable loops in the human and rat are with the framework regions of one of the flaps. A comparison of the position of CDR1 (residues 31–35, the antibody has a phenylalanine at

M. S. Neuberger, *J. Exp. Med.* **170**, 2153 (1989), and G. Winter, *Nature (London)* **321**,

Winter, *Nature (London)* **332**, 323 (1988).
 Perry, and K. S. Gottesman, "Sequences of J.S. Department of Health and Human

position 27 (phenylalanine and tyrosine are the most common amino acids at this position). Existing structural data of the human myeloma protein KOL show that Phe-27 packs against residues 32 and 34 to support CDR1.¹⁵ The NEW framework (the human acceptor for the CDR transplants of the heavy chain)¹³ has a serine at position 27 and thus fails to support the CDR in the same way as in the original rat antibody. A subsequent engineered mutation, serine to phenylalanine at position 27, restored the binding affinity of the humanized CAMPATH1 antibody close to the original affinity. This example demonstrates that amino acids in the framework, in particular those that are located close to the CDRs, must be considered. We now routinely compare the sequences of the mouse hybridoma and the human target variable sequences with each other and their respective family consensus sequences.

Here we describe the humanization of the murine monoclonal antibody BW431/26,¹⁶ which has binding specificity for carcinoembryonic antigen (CEA) and is presently used as a murine MAb for immunoscintigraphy of CEA-producing tumors such as colorectal, breast, and lung carcinomas.¹⁷

Cloning of Immunoglobulin V-Region Genes

A prerequisite for the humanization of MAb is knowledge of the nucleotide sequences of the V-region genes. To date the most elegant way to clone and sequence Ig V-region genes has been described by Orlandi and colleagues.¹⁸ They used the polymerase chain reaction (PCR)¹⁹ to amplify specifically Ig V-region genes and cloned the amplified DNA fragments into vectors that allow easy sequencing and expression. To do this they identified conserved regions at each end of the nucleotide sequences encoding V domains of mouse Ig heavy (V_H) and light (V_L) chains by comparing the frequencies of the most common nucleotides in V_H and V_L gene sequences.¹⁴ Since oligonucleotide primers used in PCR do not need to match their target sequence exactly²⁰ they were able to design oligonu-

¹⁵ M. Marquart, J. Deisenhofer, and R. Huber, *J. Mol. Biol.* **141**, 369 (1980).

¹⁶ K. Bosslet, A. Steinsträsser, A. Schwarz, H. P. Harthus, G. Lüben, L. Kuhlmann, and H. H. Sedlacek, *Eur. J. Nucl. Med.* **14**, 523 (1988).

¹⁷ R. P. Baum, A. Hertel, M. Lorenz, A. Schwarz, A. Encke, and G. Hör, *Nucl. Med. Commun.* **10**, 345 (1989).

¹⁸ R. Orlandi, D. H. Güssow, P. T. Jones, and G. Winter, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3833 (1989).

¹⁹ R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim, *Science* **230**, 1350 (1985).

²⁰ C. C. Lee, X. Wu, R. A. Gibbs, R. G. Cook, D. M. Muzny, and C. T. Caskey, *Science* **239**, 1288 (1988).

cleotides that map to the 5' and 3' regions of V_H and V_L genes and contain restriction sites for subsequent forced cloning into suitable vectors (Fig. 1 and Ref. 19).

The amplified V_H and V_L genes cover the V gene exons only. For expression in a mammalian system it is necessary to bring them into genomic configuration. The V_H and V_L genes are force cloned into KS⁺ vectors (pBluescript II KS⁺; Stratagene, La Jolla, CA) containing the 5' region of immunoglobulin heavy and light chain genes, respectively (including promoter, signal exon, and irrelevant V gene exon) as a unique *HindIII/BamHI* restriction fragment (Fig. 2). The endogenous V gene exons contain the same or compatible restriction sites as the amplification primers in the appropriate positions and are exchanged for the amplified V genes of the hybridoma by forced cloning (see the section, Cloning of V Genes). These vectors can be used for nucleotide sequence determination of the specific V genes and for forced cloning of the amplified V_H and V_L cassettes into expression vectors that contain Ig constant-region genes, using the *HindIII* and *BamHI* restriction sites (Fig. 3).

Mouse V_H Forward:

5' TGAGGAGACGGTGACCGTGGTCCCTGGCC 3'
Bst E II

Mouse V_H Backward:

5' AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG 3'
Pst I

Mouse V_K Forward:

5' GTTAGATCTCCAGCTTGGTCCC 3'
Bgl II

Mouse V_K Backward:

5' GACATTGAGCTGACCCAGTCTCCA 3'
Pvu II

FIG. 1. Oligonucleotide primer for the amplification of V_H and V_K genes. The mouse V_H backward primer is a mixture of 32 primers. The locations of the restriction sites are indicated.

V_H and V_L genes and contain
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the V gene exons only. For
necessary to bring them into
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2). The endogenous V gene
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exchanged for the amplified V
(see the section, Cloning of V
otide sequence determination
ng of the amplified V_H and V_L
tain Ig constant-region genes,
es (Fig. 3).

TCCTTGCC 3'

CAG(G/C)AGTC(T/A)GG 3'

1

CCC 3'

TCTCCA 3'

on of V_H and V_K genes. The mouse V_H
: locations of the restriction sites are

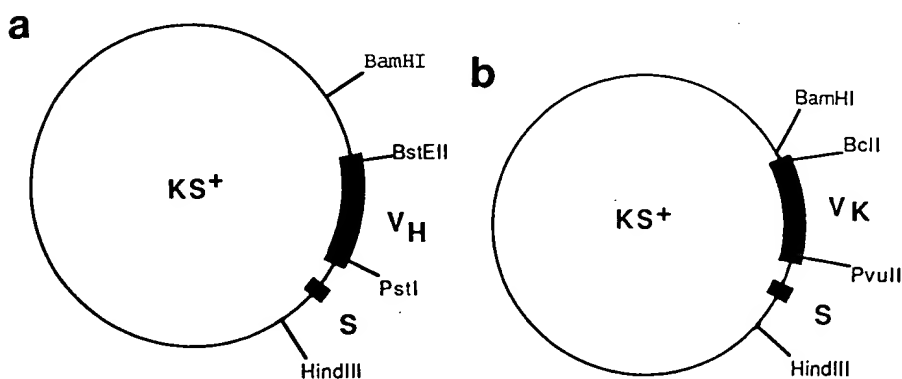


FIG. 2. Restriction maps and intron/exon organization of the V gene inserts of the KS⁺ plasmids containing a V_H (a) or a V_L (b) gene. The inserts are flanked by *Bam*HI and *Hind*III restriction sites. Exons are represented by black bars. S, Signal exon; V_H/V_K , V gene exons. The restriction sites for cloning of the amplified V genes are indicated. The *Bcl*I restriction site in the V_K exon is compatible with *Bgl*II.

Preparation of mRNA

Poly(A)⁺ mRNA is prepared following one of the standard procedures. An example is as follows:

- Centrifuge hybridoma cells (825 g; room temperature; 15 min)
- Discard supernatant
- Resuspend in 100 ml cold phosphate-buffered saline (PBS) (0°)
- Centrifuge (275 g; room temperature; 10 min)
- Discard supernatant
- Resuspend in 100 ml cold PBS
- Centrifuge as above
- Discard supernatant
- Add 22 ml lysis buffer to the pellet, vortex for 5 sec, incubate on ice (5 min)
- Centrifuge nuclei (1630 g; 4°; 15 min)
- Transfer supernatant to new tube and add 25 ml 2× proteinase K, buffer
- Incubate at 50° for 60 min
- Add 0.1 vol 5 M NaCl and let cool down to room temperature
- Apply the solution to an oligo(dT)-Sepharose column (1-ml) column volume equilibrated with binding buffer)
- Wash with 10 ml binding buffer and 15 ml washing buffer
- Elute poly(A)⁺ mRNA with 7.5 ml elution buffer and collect three fractions of 2.5 ml
- Add 0.1 vol of 3 M sodium acetate and 2.5 vol ethanol to each fraction
- Store at -20°

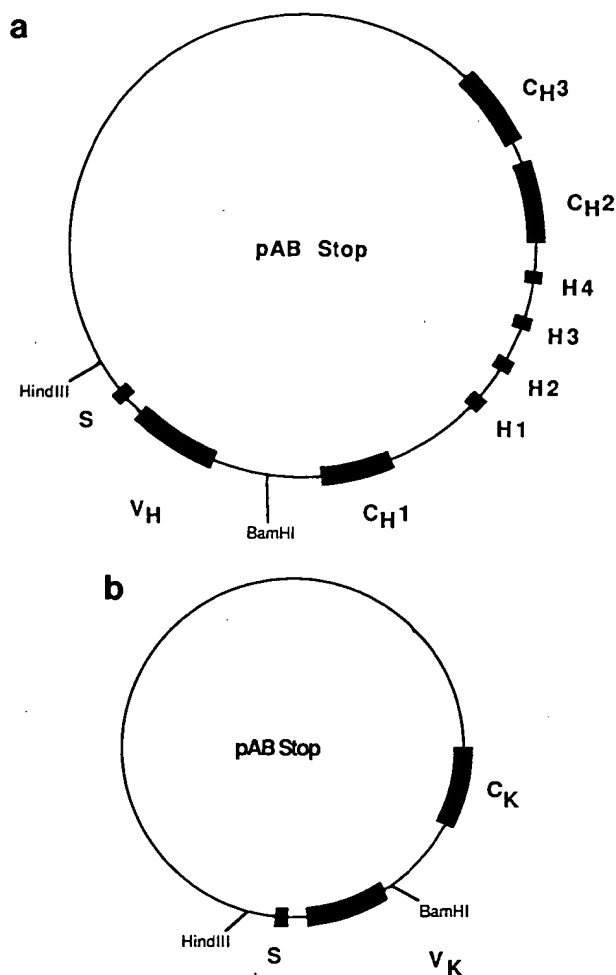
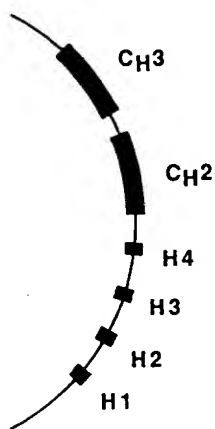


FIG. 3. Restriction maps and intron/exon organization of the expression vectors containing the human (a) heavy (IgG_3) and (b) light chain (κ) constant-region genes. The intrinsic V genes are exchanged for the hybridoma V genes using the *Hind*III and *Bam*HI restriction sites. Exons are represented by black bars. S, Signal exon; V_H/V_K , V gene exons; C_H1 , C_H2 , C_H3 , and C_K , constant-domain exons; H1, H2, H3, and H4, hinge region exons.

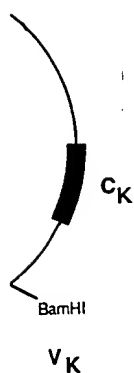
The average yield is approximately 40 μg of poly(A)⁺ mRNA per 1×10^8 hybridoma cells.

Lysis buffer: 0.14 M NaCl, 2.0 mM MgCl_2 , 10.0 mM Tris, pH 8.6, 0.5% Nonidet P-40 (NP-40)

Proteinase K buffer (2 \times): 200 mM Tris, pH 7.4, 300 mM NaCl, 25 mM



1



ion of the expression vectors contain constant-region genes. The intrinsic V g the *HindIII* and *BamHI* restriction on; V_H/V_K , V gene exons; C_H1 , C_H2 , d H4, hinge region exons.

f poly(A)⁺ mRNA per 1×10^8

, 10.0 mM Tris, pH 8.6, 0.5%

H 7.4, 300 mM NaCl, 25 mM

ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 2 mg/ml proteinase K

Binding buffer: 0.5 M NaCl, 10.0 mM Tris, pH 7.8, 5.0 mM EDTA, 0.5% SDS

Washing buffer: 0.15 M NaCl, 10.0 mM Tris, pH 7.8, 5.0 mM EDTA, 0.5% SDS

Elution buffer: No NaCl, 10.0 mM Tris, pH 7.8, 5.0 mM EDTA, 0.5% SDS

Amplification and Cloning of V Genes

Amplification of the V genes can be performed either by synthesis of first-strand cDNA followed by polymerase chain reaction (PCR) or by PCR directly from the poly(A)⁺ mRNA.²¹

First-Strand cDNA Synthesis. Mix in the following order:

Reverse transcriptase buffer (5×)	10.0 μ l
Dithiothreitol (DTT) (0.1 M)	2.5 μ l
Human placental ribonuclease inhibitor (50 U/ μ l)	3.0 μ l
dNTPs (10 mM)	5.0 μ l
Primer (10 pM/ μ l)	5.0 μ l
Poly(A) ⁺ mRNA (5–10 μ g)	
ddH ₂ O	to 47.0 μ l

Mix gently and centrifuge for a few seconds, then add

Reverse transcriptase (25 U/ μ l)	3.0 μ l
---------------------------------------	-------------

Incubate at 42° for 1 hr.

PCR Reaction from cDNA

H ₂ O:	29.5 μ l
dNTPs (2.5 mM):	5.0 μ l
PCR buffer (10×):	5.0 μ l
Oligonucleotide primers (5 pM/ μ l):	5.0 μ l
Taq polymerase (The Perkin-Elmer Corp., Emeryville, CA) (2.5 U/ μ l) ^{21a}	0.5 μ l

At this point the reaction mix can be treated for 5 min with UV light (254 or 300 nm) to avoid contamination (for example, with previously amplified DNA).

²¹ W. T. Tse and B. G. Forget, *Gene* 88, 293 (1990).

^{21a} In case of the V_H primers the Taq polymerase was added to the reaction mixture after the first denaturation and annealing steps to avoid primer dimer formation.

Add 5.0 μ l of first-strand cDNA synthesis reaction and seal the surface of the reaction mixture with a drop of paraffin oil. Amplify for 30 to 50 cycles. The standard programs we use are the following: 94°, 1 min; 52° (for V_K , V_L) or 57° (for V_H), 1 min; 72° for 2 min.

Analyze 5 μ l of the reaction mixture on a 2% agarose gel.

PCR Reaction from mRNA. For PCR for mRNA resuspend 1–5 μ g of poly(A)⁺ mRNA in 50 μ l PCR reaction mix and proceed as described above for the PCR from cDNA.

If the amplification reactions are unsuccessful under these conditions, the addition of 5.0 μ l Perfect Match (Stratagene) and a modification of the annealing and extension conditions as well as $MgCl_2$ concentrations may improve the results.

Cloning of V Genes

If the amplification is successful, the remaining 45 μ l of the reaction mixture is purified with GeneClean (BIO 101, Inc., La Jolla, CA). The purified amplification products (in 20 μ l doubly distilled H_2O) are treated as follows: 10 μ l is double digested with *Pst*I/*Bst*EII for the V_H gene or *Pvu*II/*Bgl*II for the V_L gene. The digested samples are analysed on a 2% TAE agarose gel with 3 μ l of undigested amplification product as control. The residual amplification product is stored at –20° as the template for the synthesis of additional material, if required.

The vectors for cloning the amplified V genes have been constructed by isolation of the *Hind*III/*Bam*HI inserts of the M13 VHPCR and M13 VKPCR vectors described in Orlandi *et al.*¹⁸ into KS⁺ plasmids. Two internal *Pvu*II sites were removed from the KS⁺ plasmid backbone prior to the construction of the KS⁺ VKPCR vector (C. Weber, unpublished data, 1990).

These KS⁺ vectors (KS⁺ VHPCR and KS⁺ VKPCR) are digested with *Pst*I and *Bst*EII (V_H) or *Pvu*II and *Bcl*I (V_L), respectively, and the vector fragment is purified on a 0.8% agarose gel.

Note: The KS⁺ VKPCR vector was prepared in *Escherichia coli* JM110 (ATCC No. 47013) to avoid dam methylation of the *Bcl*I site.

From the resulting colonies DNA minipreparations are performed and the size of the inserts is analyzed by a *Hind*III/*Bam*HI digestion. Clones containing inserts of the correct size (approximately 800 bp for V_H and 650 bp for V_L) are sequenced²² using primers based 3' of the CDR3 regions of the V genes (Fig. 4).

Since hybridoma cells may produce more than one heavy or light chain and can contain additional nonfunctional mRNAs it is advisable to clone the isolated V genes into appropriate expression vectors containing Ig C_H

²² F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).

sis reaction and seal the surface with paraffin oil. Amplify for 30 to 50 cycles the following: 94°, 1 min; 52° or 2 min.

Run on a 2% agarose gel. Extract total RNA or mRNA resuspend 1–5 µg of total RNA in mix and proceed as described.

Successful under these conditions, (gene) and a modification of the protocol as MgCl₂ concentrations may

remaining 45 µl of the reaction mixture (Boehringer-Mannheim, Inc., La Jolla, CA). The doubly distilled H₂O are treated with PstI/BstEII for the V_H gene or HindIII/BstEII for the V_L gene. 1 sample is analysed on a 2% agarose gel. The amplification product as control. Run at –20° as the template for sequencing.

V genes have been constructed by cloning of the M13 VHPCR and M13 VLPCR into KS⁺ plasmids. Two samples of KS⁺ plasmid backbone prior to cloning or (C. Weber, unpublished data,

KS⁺ VKPCR) are digested with HindIII/BstEII, respectively, and the vector

prepared in *Escherichia coli* JM110 for ligation of the BclI site.

Preparations are performed and HindIII/BamHI digestion. Clones of approximately 800 bp for V_H and 650 bp for V_L based 3' of the CDR3 regions of

more than one heavy or light chain mRNA it is advisable to clone expression vectors containing Ig C_H

Int. Acad. Sci. U.S.A. 74, 5463 (1977).

V_K/V_L: 5' GGA TCC AAC TGA GGA AGC 3'

V_H: 5' TGT CCC TAG TCC TTC ATG ACC T 3'

FIG. 4. Oligonucleotides for sequencing of the cloned V genes.

and C_L genes and express them in eukaryotic cells (see section entitled "Expression of Humanized MAb"). The resulting chimeric Ig molecules contain the complete mouse V domains and should have the identical antigen-binding properties as the original mouse MAb, if the right pair of V_H and V_L genes has been amplified and cloned.

Reverse transcriptase buffer (5X): 250 mM Tris, pH 8.3, 30 mM MgCl₂, 500 mM NaCl

PCR buffer (10X): 100 mM Tris, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% (w/v) gelatin

TAE buffer (50X): 2 M Tris base, 1 M acetic acid, 50 mM EDTA

The solutions are autoclaved before use, distributed in 0.5-ml aliquots, and stored at –20°.

Design of Oligonucleotides for Mutagenesis

After determination of the nucleotide sequences of the hybridoma V genes (Fig. 5) they are translated into the corresponding amino acid sequences and compared to the consensus sequences of the murine Ig heavy and light chain V gene families to identify their families of origin (Fig. 6).

Comparison of the translated BW431/26 V_H sequence with the mouse V_H consensus sequences marks it as a member of family IA. Likewise BW431/26 V_L is identified as a member of family VI of the κ light chains.

For designing the reshaped V regions, it is necessary to identify amino acid residues that are not conserved or only semiconserved in the framework regions of the human and mouse antibodies in question. Framework regions that are not conserved between human and mouse antibodies, but which might help to shape the CDRs or which might interact directly with the antigen, have to be identified. Unusual amino acid replacements are changes from hydrophilic to hydrophobic, large to small, or change in electrostatic charge. The following amino acid exchanges are considered as conserved replacements and are excluded from the computer inspection: F - Y, L - M, V - I, Q - H, D - E, R - K, W - R, N - D, G - A, S - T, Q - D/E, H - R/N, I - L/M, L - F/V, and V - L/M. The framework and CDR regions are defined by Kabat *et al.* as shown in Fig. 7.

The decision whether framework exchanges must be introduced in addition to the CDR grafting is then made by inspection of the location of the identified amino acid residues in the acceptor V_H or V_K three-dimensional structure.

a

10 30 50
L Q E S G P D L V K P S Q S L S L T C T
ctgcaggagtcaggacctgacctggtgaaaccttctcagtcactttcactcacctgcact

70 90 110
V T G Y S I T S G Y S W H W I R Q F P G
gtcactggctactccatcaccAGTGGTTATAGCTGGCACTggatcoggcagtttccagga

130 150 170
N K L E W M G Y I Q Y S G I T N Y N P S
aacaaactggaatggatgggcTACATACAGTACAGTGGTATCACTAACTACAACCCCTCT

190 210 230
L K S R I S I T R D T S K N Q F F L Q L
CTCAAAAGTogaatctctatcactcgagacacatccaagaaccagttcttctcagcttg

250 270 290
N S V T T E D T A T Y Y C A R E D Y D Y
aattcagtgactactgaggacacagccacatattactgtgcaagaGAAGACTATGATTAC

310 330 350
H W Y F D V W G A G T T V T V S S
CACTGGTACTTCGATGTCtgggcgagggaccacgggtcacogtctcctca

b

10 30 50
Q L T Q S P A I M S A S L G E E I T L T
cagctgaccagctctccagcaatcatgtctgcatctctaggggaggagatcacctaacc

70 90 110
C S T S S S V S Y M H W Y Q Q K S G T S
tgAGTACCAGCTGAGTGTAGTTACATGCACTggtaggcagagtcaggcacttct

130 150 170
P K L L I Y S T S N L A S G V P S R F S
cccaaaactcttgatattatAGCACATCCAACCTGGCTTCTTggagtccttctcgttctcag

190 210 230
G S G S G T F Y S L T I S S V E A E D A
ggcagtgaggctctgggacctttattctctcacaatcagcagtgaggaggtgaagatgct

250 270 290
A D Y Y C H Q W S S Y P T F G G G T K L
gccgattattactgcCATCAGTGGAGTAGTTATCCAGTtcggaggggggaccaagctg

E I
gagatca

FIG. 5. Nucleotide sequences of the murine BW431/26 (a) V_H and (b) V_K genes. The sequences start with the *Pst*I/*Pvu*II restriction sites and continue to the end of the V exon. CDR sequences are typed in capital letters. The amino acid sequences are given in the single-letter code on top of the first base of the triplets.

50

Q S L S L T C T
ctcagtcactttcactcacctgcact

110

H W I R Q F P G
GGCActggatccggcagtttccagga

170

G I T N Y N P S
AGTGGTATCACTAACTACAAOCCCTCT

230

K N Q F F L Q L
ccaagaaccagttcttctcgcagttg

290

C A R E D Y D Y
tactgtgcaagaGAAGACTATGATTAC

350

T V T V S S
acggtcacogtctcctca

50

S L G E E I T L T
atctctaggggagagatcacctaacc

110

W Y Q Q K S G T S
Ctggtagcagcagaagtcaggcacttct

170

A S G V P S R F S
GGCTTCTggagtcacctctcgttcagt

230

I S S V E A E D A
aatcagcagtgaggagctgaagatgct

290

P T F G G G T K L
ATCCACGttcggaggggggaccaagctg

; BW431/26 (a) V_H and (b) V_K genes. The
ites and continue to the end of the V exon.
he amino acid sequences are given in the
iplets.

A three-step comparison of the framework amino acid residues consists of (1) the donor (mouse) antibody V region with its own V region family consensus sequence, (2) the acceptor (human) antibody V region with its own V region family, and (3) the mouse and human families, which should allow the differences to be dissected in a systematic way, identifying those differences peculiar to the individual antibodies or their entire families.

Heavy Chain V_H

Step 1. The sequence of the (mouse) donor is compared with the consensus sequence for its own family of V_H genes (Fig. 8).

Step 2. The sequence of the (human) acceptor is compared with the consensus sequence for its own family of V_H genes (human V_H II; HuVH.CON2 in Fig. 9).

Step 3. The consensus sequences of the donor (mouse) and acceptor (human) V_H families are compared (Fig. 10).

Light Chain V_K

Step 1. The sequence of the (mouse) donor is compared with the consensus sequence of its own V_K family (MOVK VI) (Fig. 11).

Step 2. The sequence of the (human) acceptor is compared with the consensus of its own V_K family. The human REI V_K sequence is based on Ref. 13. REI is a member of the human V_K family I (HuVKCON SUB1 in Fig. 12).

Step 3. The consensus sequences of the acceptor (human) and donor (mouse) V_K families are compared (Fig. 13).

All unusual amino acid replacements that occur outside the CDRs, as defined by Kabat *et al.*,¹⁴ are marked and the positions at which replacements occur are inspected by computer graphics (Evans & Sutherland Corp., Salt Lake City, UT; Frodo²³) using as a basis the X-ray structures of NEW²⁴ and REI.²⁵ All positions in question are highlighted with a 200% van der Waals radius, to see whether the amino acid residue at a certain position is close to, or actually makes contact with, any of the amino acids composing the CDRs. In such a case an amino acid replacement in the framework of the human V_H or V_K region must be considered.

²³ T. A. Jones, in "Computational Crystallography" (D. Sayre, ed.), p. 303. Oxford Univ. Press (Clarendon), London and New York, 1982.

²⁴ R. J. Poljak, L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul, *Proc. Natl. Acad. Sci. U.S.A.* 70, 3305 (1973).

²⁵ O. Epp, P. Colman, H. Fehlhhammer, W. Bode, M. Schiffer and R. Huber, and W. Palm, *Eur. J. Biochem.* 45, 513 (1974).

a		10	20	30	40	50
1	MOVH IA.CON	EVQLQESGPSLVKPSQTL	SLTCSVTGDSITS	SDYWNXXWIRQ	FPGNKLEWM	
2	MOVH IB.CON	QVQLKESGGLVAPSQSL	SITCTVSGFSLTSY	GVHXSWMRQ	PPGKGLGTL	
3	MOVH IIA.CON	EVQLQQSGPELVKPGAS	VKMSCKASGYTFTD	YYMXXWVKQ	SHGKSLEWI	
4	MOVH IIB.CON	QVQLQQPGAEVLKPGAS	VKLSCKASGYTFTS	YMMXXWVKR	PPGQGLEWI	
5	MOVH IIC.CON	EVQLQQSGAEVLKPGAS	VKLSCTASGFNIKDT	YMMXXWVKR	PPGQGLEWI	
6	MOVH IIIA.CON	EVKLVESSGGLVQPGSL	RSLSCATSGFTSDF	YMMXXWVKR	PPGKRLLEWI	
7	MOVH IIIB.CON	EVKLLESGGLVQPGSL	KLSCAASGFDTSRY	MMXXWVKR	PPGKRLLEWI	
8	MOVH IIIC.CON	EVYLEESSGGLVQPGS	MYLSCVASGFTSN	YMMXXWVKR	PPGKRLLEWI	
10	MOVH VA.CON	EVQLQQSGAEVLVAGSS	VKMSCKASGYTFTS	YGINXXWVKR	PPGQGLEWI	
11	MOVH VB.CON	EVQLQQSGAEVLKAGSS	VKMSCSATGYTSS	YGLYXXWVKR	PPGQGLEXX	
	CONSENSUS	-V-L--G--L-----	C--G-----	W-----	L---	
		60	70	80	90	100
1	MOVH IA.CON	GYISNXXYSGSTYYN	PSLYSRISITRDT	SKNFFLQLNS	VTTEDTATYYC	
2	MOVH IB.CON	GVIVXXXAGGSTNYN	SALMSRLSISKD	NSKSQVFLK	MNSLQTD	TAMYYC
3	MOVH IIA.CON	GDINPXXNNGGTSYN	QKFKGKATLTVD	KSSSTAYMQL	NLSLTS	SEDSAVYYC
4	MOVH IIB.CON	GRIDPXXNSGGTKY	NEKFKSKATLTVD	KSSSTAYMQL	NLSLTS	SEDSAVYYC
5	MOVH IIC.CON	GRIDPXXANGNTKY	DPKFGKATITAD	TSSNTAYLQL	NLSLTS	SEDTAVYYC
6	MOVH IIIA.CON	AASRNKANDYTTE	YSASVKGRTIVSR	DTSSILYLQ	MNALRAED	TATYYC
7	MOVH IIIB.CON	GEINPKADSS	TINYTPSLYDYFI	ISRDNAKNTLY	LQMSYVR	SEDTALYYC
8	MOVH IIIC.CON	AEIRLYSHNYATH	YAESVYGRFTISR	DDSYSSVYLQ	MNALRAED	TGIYYC
10	MOVH VA.CON	GYINPXXNGYTKY	NEKFKGKTTLTVD	KSSSTAYMQL	NLSLTS	SEDSAVYFC
11	MOVH VB.CON	GYISSXXSSAYP	NYAQKFGRTITAD	ESTNTAYMEL	SSLRSEDT	AVYFC
	CONSENSUS	-----Y-----	D-----	D-----	D--Y-C	
		110	120	130	140	150
1	MOVH IA.CON	ARLYGYRGDEEDY	YAMAFDYWGQ	GTTXTVSS		
2	MOVH IB.CON	ARDRGVXRYPDKY	FTLWFDYWGQ	GTLTVSS		
3	MOVH IIA.CON	ARDYYWYFXXXXXXXX	YWFYWGQ	GTTTVSS		
4	MOVH IIB.CON	ARYXYGSSSGYXXY	YFDYWGQ	GTTTLTVSS		
5	MOVH IIC.CON	ARGYXXYDXXXXXXXX	YAMDYWGQ	GTSVTVSS		
6	MOVH IIIA.CON	ARDYYGSSXTFGXX	YWFYDVWGAG	TTVTVSS		
7	MOVH IIIB.CON	ARLYGYGYFGSSXX	YXXAYWGQ	GTTTVTVSS		
8	MOVH IIIC.CON	TTGFVPXXXXXXXXXX	YAYWGQ	GTLTVTVSS		
10	MOVH VA.CON	ARSNYGGSYFFXXXX	YFDYWGQ	GTTTLTVSS		
11	MOVH VB.CON	AVRVISRYFXXXXXX	YDYGQ	GTLV		
	CONSENSUS	-----WG-GT--TVSS				

FIG. 6. Consensus sequences of the murine (a) V_H and (b) V_K gene families are listed in the single-letter code. The amino acid positions that are conserved among all V_H/V_K gene families are outlined in the bottom lines.

30 40 50
 /TGDSITSYNDXQWIRQFPNGKLEWM
 /SGFSLTSYGVHXSQWVRQPPGKGLGTL
 ASGYTFTDYVMXQXWVKQSHGKSLLEWI
 ASGYTFTSYVMXQXWVKQRPQGGLLEWI
 ASGFNIKDTYMHXQXWVKQRPQGGLLEWI
 TSGFTFSDFYMEXQXWVRQPPGKRLLEWI
 ASGFDFSRYSXQXWVRQAPGKGLLEWI
 ASGYTFTSYVMXQXWVRQSPGYGLEWV
 ASGYTFTSYGINDXWVKQRPQGGLLEWI
 ATGYTFTSSYGLYXQXWVRQAPGQGLEXX
 ---G-----W-----L---

80 90 100
 IITRDTSKNQFFLQNSVITTEDTATYYC
 IISKDNKSQVFLKXNSLQTDDTAMYYC
 ILTVDKSSSTAYMQLNSLTSEDSAVYYC
 ILTVDKSSSTAYMQLNSLTSEDSAVYYC
 ITADTSSNTAYLQLSSTSEDTAVYYC
 IVSRDTSQSLIYLQMNALRAEDTATYYC
 IISRDNAKNTLYLQMSYVRSEDTALYYC
 TISRDDSYSSVYLQMNALRAEDTGIYYC
 ILTVDKSSSTAYMQLNSLTSEDSAVYFC
 TITADESTNTAYMELSSLRSEDTAVYFC
 ---D-----D---Y-C

130 140 150
 IGQGTXTTVSS
 IGQGTTLTVSS
 IGQGTTVTVSS
 IGQGTTLTVSS
 IGQGTSTTVSS
 IGAGTTTVSS
 IGQGTTVTVSS
 IGQGTTLTVSS
 IGQGTILV
 IG-GT--TVSS

and (b) V_K gene families are listed in the
 are conserved among all V_H/V_K gene

b

10 20 30 40 50
 1 MOVK I.CON DIVMTQSPSSSLAVSAGEKVTXSCTASESLYSSKHVHYLAWYQKKPEQS
 2 MOVK II.CON DVVMTQTPLSLPVSLGDAQISCRSSQSLVHSXNGNTYLNWYLQKPGG
 3 MOVK III.CON DIVLTQSPASLAVSLGQRATISCRASESVDXXXXYGNSPMHWYQKPGQPP
 4 MOVK IV.CON EXVLTQSPAIMAASPGKEVTMTCSASSXXXXXVSSYLHWYQKPGGASP
 5 MOVK V.CON DIQMTQSPSSLSASLGDRVTITCRASQXXXXXXDISNYLNWYQKPGGTP
 6 MOVK VI.CON QIVLTQSPAIMASPGKEVTMTCSASSXXXXXSVSYMHYQKPGGTSP
 7 MOVK.MBR DIQLTQSPPSLTVSVGERVTISCKSNQNLWSGNRRYCLGWHWKPGQTP
 CONSENSUS ----TQ-P-----S-G-----C-----W---K----P
 60 70 80 90 100
 1 MOVK I.CON KLLIYGASNRYIGVPDRFTGSGSGTDFTLTISVQVEDLTHYYCAQFYYSY
 2 MOVK II.CON KLLIYKVSNRFSGVDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGTHTV
 3 MOVK III.CON KLLIYAASNLGVPARFSGSGSGTDFTLNHPVEEDDAATYYCQGSNED
 4 MOVK IV.CON KLXIYXTNLSAGVPARFSGSGSGTSYSLTISSEAEDDATYYCQQWSGY
 5 MOVK V.CON KLLIYASRLHSGVPSRFSGSGSGTDYSLTISSELEZEDATYYCQQGNLSL
 6 MOVK VI.CON KRWIYDTSKLASGVPARFSGSGSGTSYSLTISSEAEADAATYYCQQWSSN
 7 MOVK.MBR TPLITWISDRFSGVDRFIGSGSVTDFTLTISVQAEADVAVYFCQQHLDL
 CONSENSUS ---I---S---GVP-RF-GSGS-T---L-I-----D---Y-C-Q---
 110 120 130 140 150
 1 MOVK I.CON PXXXXXXLTFGAGTKLELYRX
 2 MOVK II.CON PXXXXXXYTFGGGKLEIKRA
 3 MOVK III.CON PXXXXXXTFGGGKLEIKRA
 4 MOVK IV.CON PFXXXXXXTFGXGKLEIKRX
 5 MOVK V.CON PXXXXXXRTFGGGKLEIKRA
 6 MOVK VI.CON PPMXXXXLTFGAGTKLEIKRX
 7 MOVK.MBR PXXXXXXYTFGGGKLEI
 CONSENSUS P-----TFG-GTKLE-----A

Fig. 6b.

	V_H	V_K
FR 1	1-30	1-23
CDR1	31-35	24-34
FR2	36-49	35-49
CDR2	50-65	50-56
FR3	66-94	57-88
CDR3	95-102	89-97
FR4	103-113	98-107

Fig. 7. Definition of CDR regions according to Kabat *et al.*¹⁴

	10	20	30	35ab	40
MOVH IA	EVQLQESG	PSLVKPSQ	TLSTCTSV	TGDSITSD	YWNXXWIRQ
BW431/26VH	QVQLQESG	PDVLPKPS	QSLSLTCT	VTGYSITSG	YSWH-WIRQ
	*	*	*	*	*
	52abc	60	70	80 abc	90
MOVH IA	GYISNXXY	SGSTYYNP	SLYSRISIT	RDTSKNQF	FLQLNSVT
BW431/26VH	GYI----	YSGITNYP	SLKSRSIT	RDTSKNQF	FLQLNSVT
	100abcde	fghijk	110		
MOVH IA	ARLYGYRG	DEEDYYAM	AFDYWGQ	GTTXTVSS	
BW431/26VH	AR-----	EDYDHW	FVWGAGT	TVTIVSS	

FIG. 8. Comparison of BW431/26 V_H sequence with the mouse V_H IA (MOVH IA) consensus sequence. Relevant framework differences are marked with an asterisk. The differences are as follows: EQ1, SD10, TS17, ST23, DY27, QA105. X represents amino acids that are variable in the consensus sequence. These are not considered as exchanges. Dashes in the BW431/26 sequence are introduced to accommodate for the variable length of the CDRs.

In the case of the BW431/26 antibody, for V_H the exchange of a phenylalanine for a serine at position 27, and that of an isoleucine for a phenylalanine at position 29 in the NEW V_H gene have been considered as additional framework exchanges. With the exchange of the amino acid at position 27 from serine to phenylalanine we followed Riechmann's strategy for the humanization of the CAMPATH 1 antibody.¹³ Phe-27 is present in most human V_H domains and may play an important role in positioning CDR1. Since Phe-27 is changed to serine in the NEW V_H domain it seems necessary to introduce Phe-27 into the humanized V_H .

Phe-29 of NEW in the immediate vicinity of CDR1 contains an aro-

	10	20	30	35ab	40
HuVH.NEW	QVQLQESG	PGLVRPSQ	TLSTCTSV	SGSTFSD	YYSTXXWVR
HuVH.CON2	XVTLRESG	PXLVKPTET	LTLTCTV	SGFSLSTX	GMXGVGWI
	*	*	*	*	*
	52abc	60	70	82abc	90
HuVH.NEW	GYVFIHGT	SDTDPLRS	XXXRVTML	VDTSKNQF	SLRLSSVT
HuVH.CON2	ARINXXXW	DDDKYYST	SLRSRLTI	SYDTSKNQ	VVLXXXXD
	*	*	*	*	*
	100abcde	fghijk	110		
HuVH.NEW	ARNLIAGC	IXXXXXXXXX	XDVGQGS	LTVTVSS	
HuVH.CON2	ARRXPRXX	GDXGXYYX	AFDVWGQ	GTTTVTVSS	

FIG. 9. Comparison of the NEW V_H sequence with the human V_H II (HuVH.CON2) family consensus sequence. The NEW V_H sequence is based on Ref. 13. Framework differences are marked with an asterisk. The differences are as follows: QT3, QR5, SF27, FL29, DT31, YR50, FN52, TW53, SD54, DK57, TY58, PY59, LS60, RT61, VL67, LS70, VY71, FV78, SV79, TD83, AP84, VT89, NR95, IP97, AR98, LT109.

30 35ab 40
VTGDSITSYWNXXWIRQPPGNKLEWM
VTGYSITSGYSWH-WIRQPPGNKLEWM
*

70 80 abc 90
ITRDTSKNQFFLQLNSVTEDTATYYC
ITRDTSKNQFFLQLNSVTEDTATYYC

110
QGTTXTVSS
AGTTTVTVSS
*

ice with the mouse V_H IA (MOVH IA) es are marked with an asterisk. The differ-27, QA105. X represents amino acids that not considered as exchanges. Dashes in the ate for the variable length of the CDRs.

body, for V_H the exchange of a 7, and that of an isoleucine for a V_H gene have been considered as the exchange of the amino acid at e we followed Riechmann's strat-IPATH 1 antibody.¹³ Phe-27 is d may play an important role in nged to serine in the NEW V_H Phe-27 into the humanized V_H . icinity of CDR1 contains an aro-

30 35ab 40
TVSGSTFSYYSTXWVRQPPGRGLEWI
TVSGFSLSTXGMXVGWIRQPPGKXLEWL
* * *

70 82abc 90
TMLVDTSKNQFSLRLSSVTAADTAVYYC
TISYDTSKNQVVLXXXXDPXDTATYYC
* * * * *

110
QGGLVTVSS
QGTTTVTVSS
*

ce with the human V_H II (HuVH.CON2) ice is based on Ref. 13. Framework differ- s are as follows: QT3, QR5, SF27, FL29, , PY59, LS60, RT61, VL67, LS70, VY71, R98, LT109.

	10	20	30	35ab	40
HuVH II	-VTRESGPXLVYPTETLTTLTCTVSGFSLSTXGMXVGWIRQPPGKXLEWL				
MOVH IA	EVQLQESGSPSLVKPSQTLSTLTCVSGFSLSTXGMXVGWIRQPPGKXLEWL				
	* *	* *	*		* *
	52abc	60	70	80 abc	90
HuVH II	ARINXXXWDDDKYYSTSLRSRLTISYDTSKNQVVLXXXXDPXDTATYYC				
MOVH IA	GYISNXXYSGSTYYNPSLYSRISITRDTSKNQFQLQLNSVTEDTATYYC				
			*	**	***
	100abcde fgh i j k	110			
HuVH II	ARRXPRXXXGDXGXYYXAFDVWGQGTITVTVSS				
MOVH IA	ARLYGYRGDEEDYYAMAFDYWGQGTITVTVSS				
	* * *	* * *	*		

FIG. 10. Comparison of the acceptor (human V_H II; HuVH II) and the donor mouse V_H IA; MOVH IA) consensus sequences. Relevant differences in the framework regions are marked with an asterisk. Differences between donor and acceptor consensus sequences are as follows: DEL E1, TQ3, RQ5, YK13, EQ16, FD27, PF40, KN43, YR71, VF78, VF79, XQ81, XS82C, XV83, DT84, PT85, XE86, RL95, XY96 (X = T, C, V, P, Q, H, R, L, N), PG97, RY98, XY99 (X = P, Q, V, R, M, T, G), DE100C, XE100D (X = Y, L, M, G, V), GD100E, XA100H (X = N, S, D, Y), XM100I (X = S, D, G), VY102.

	10	20	27abcde f	30	40
MOVK VI	QIVLTQSPAIMASAPGKVTMTCSASSXXXXXXXXXSVSYMHWYQQKPGSSP				
BW431/26VK	-----AIIASAPGKVTMTCRASSXXXXXXXXXSVSYMHWYQQKPGSSP				*
	50	60	70	80	90
MOVK VI	KRWLYDTSKLASGVPARFSGSGSGTSYSLTISSEAEADAATYYCQWSSN				
BW431/26VK	KRWLYATSNLASGVPARFSGSGSGTSYSLTIIRVEADAATYYCQWSSN				**
	95abcde f	100	106a		
MOVK VI	PPMXXXLTFGAGTKLELXKR				
BW431/26VK	PXXXXXXLTFGAGTKLEIX---				

FIG. 11. Comparison of the 431/26 V_K sequence with the mouse V_K VI (MOVK VI) family consensus sequence. The relevant differences in the framework regions are marked with an asterisk. The differences are as follows: SP40, RP46, SI76, SR77.

	10	20	27abcde f	30	40
HuVKCON SUB1	DIQMTQSPSSLSASVGDRTVITCRASQSVXXSXDISSYLNWYQQKPGKAP				
HuVK REI	DIQMTQSPSSLSASVGDRTVITCQASQ-----DISDYLNWYQQKPGKAP				
	50	60	70	80	90
HuVKCON SUB1	KLLIYXASSLESQVPSRFSGSGSGTDFTLTISLQPEDFATYYCQYNSL				
HuVK REI	KLLIYEASNLQAGVPSRFSGSGSGTDFTFITISLQPEDATYYCQYQSL				*
	95abcde f	100	106a		
HuVKCON SUB1	PXXYDXXYTFGQGTKEIKRT				
HuVK REI	P-----YTFGQGTKEI-KR-				

FIG. 12. Comparison of the human acceptor sequence REI with the consensus sequence of the human V_K I family (HuVKCON SUB1). The relevant framework difference is marked with an asterisk; the difference is F183.

		10	20	27abcdef	30	40
HuVKCON SUB1		DIQMTQSPSSLSASVGD	RVITTCRASQSVXXSX	DISSYLNWYQQKPGKAP		
MOVK VI		QIVLTQSPAIMSASPGEKVTMTCSASSXXXXXXSVSYMHYQQKSGTSP				
		*	**	*		**
		50	60	70	80	90
HuVKCON SUB1		KLLIYKASSLESGVPSRFSGSGSGTDFTLT	TISSLPEDFATYYCQYNSL			
MOVK VI		KRWIYDTSKLASGVPARFSGSGSGTSYSLT	ISSMEADAATYYCQWSSN			
		**	*	*	*	*
		95abcdef	100	106a		
HuVKCON SUB1		PXXYDXXYTFGQGTKVEIKRRT				
MOVK VI		PPMXXXXLTFGAGTKLELXKR				
			*			

FIG. 13. Comparison of the consensus sequences of the acceptor (HuVKCON SUB1) and donor (MOVK VI) V_K families. Relevant differences in the framework regions are marked with an asterisk. The differences are as follows: QV3, SA9, SI10, VP15, PS41, KT43, AS44, LR47, LW48, SA60, DS70, PA80, FA83, QA100.

matic ring and is larger than Ile-29 of BW431/26. Therefore it might interact with CDR1 side chains in a different way than does Ile-29, so we decided to use Ile-29 in the humanized MAb. For the light chain no additional exchanges were necessary.

On the basis of these data the oligonucleotides for the mutagenesis of the human V genes were designed. The oligonucleotides span the CDR coding region, including additional exchanges in the adjacent frameworks, and at their 5' and 3' regions they contain 12 bp that is complementary to the flanking framework regions of the human V genes. The CDR1 oligonucleotide for the light chain had to be placed differently (Fig. 14).

For the VK CDR1 oligonucleotide the overlap with the frameworks at its 3' and 5' ends had to be shortened to 9 b. The full-length (12-b overlap) oligonucleotide exhibited cross-hybridization with framework three sequences and this gave rise to misincorporation during mutagenesis.

Mutagenesis of Human V Genes

The mouse CDRs are placed into the human frameworks by oligonucleotide-directed mutagenesis. The mutagenesis of the human V genes can be performed with any mutagenesis system available. We use the gapped duplex (gd) DNA method and the pMa/c phasid vectors described by Stanssens and colleagues.²⁶ The gapped duplex method routinely yields a

²⁶ P. Stanssens, C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H.-J. Fritz, *Nucleic Acids Res.* 17, 4441 (1989).

27abcdef 30 40
 FITCRASQSVXXSXDISSYLNNWYQKPGKAP
 IMTCSASSXXXXXXXXSVSYMHWYQKSGTSP
 * **

70 80 90
 GSGSGTDFTLTISSLPEDFATYYCQYNSL
 GSGSGTSYSLTISSMEADAATYYCQWSSN
 * * *

KRT
 KRK

ences of the acceptor (HuVKCON SUB1) and
 rences in the framework regions are marked
 QV3, SA9, SI10, VP15, PS41, KT43, AS44,
 0.

of BW431/26. Therefore it might
 different way than does Ile-29, so we
 ized MAb. For the light chain no

gonucleotides for the mutagenesis of
 The oligonucleotides span the CDR
 changes in the adjacent frameworks,
 tain 12 bp that is complementary to
 e human V genes. The CDR1 oligo-
 e placed differently (Fig. 14).
 e the overlap with the frameworks at
 to 9 b. The full-length (12-b overlap)
 idization with framework three se-
 rporation during mutagenesis.

the human frameworks by oligonu-
 mutagenesis of the human V genes can
 system available. We use the gapped
 pMa/c phasmid vectors described by
 ed duplex method routinely yields a

vn, W. Kramer, M. Zabeau, and H.-J. Fritz,

VK - CDR1

5' CTG GTA CCA GTG CAT GTA ACT TAC ACT CGA GCT GGT
 ACT ACA GGT GAT 3'

VK - CDR2

5' GCT TGG CAC ACC AGA AGC CAG GTT GGA TGT GCT GTA GAT
 CAG CAG 3'

VK - CDR3

5' CCC TTG GCC GAA CGT GGG ATA ACT ACT CCA CTG ATG GCA
 GTA GTA GGT 3'

VH - CDR1

5' CTG TCT CAC CCA GTG CCA GCT ATA ACC ACT GCT GAT GGT
 GAA GCC AGA CAC GGT 3'

VH - CDR2

5' CAT TGT CAC TCT ACT TTT GAG AGA GGG GTT GTA GTT AGT
 GAT ACC ACT GTA CTG TAT GTA TCC AAT CCA CTC 3'

VH - CDR3

5' GCC TTG ACC CCA GAC ATC GAA GTA CCA GTG GTA ATC
 ATA GTC TTC TCT TGC ACA ATA 3'

FIG. 14. Oligonucleotides for the CDR exchange mutagenesis. The bases originated from the BW431/26 sequence and the codon for Phe-27 in V_H are printed in bold letters.

larger percentage of recombinant clones with all three CDRs mounted in a single step (5–20%), as compared with classical M13 mutagenesis.¹³ The template for mutagenesis of the V_H gene is the human myeloma protein NEW and for the V_L gene it is human myeloma protein REI, or any CDR-grafted version of these genes. We used the humanized versions containing the CDR regions of the anti-lysozyme MAb D1.3²⁷ (Fig. 15a and b).

²⁷ M. Verhoeven, C. Milstein, and G. Winter, *Science* 239, 1534 (1988).

a

10 30 50
aagcctatgaatatgcaaatcctgctcatgaatatgcaaatcctctgaatctacatggta
Hind 3

70 90 110
aatatagggtttgtctataccacaacagaaaaacatgagatcacagttctctctacagtt

130 150 170
M G W S C I I L F L V A T
actgagcacacaggacctcaccATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAAC

190 210 230
A T
AGCTACAggtaaggggctcacagtagcaggcttgaggctcggacatatatatgggtgaca

250 270 290
G V H S Q V Q L Q E
atgacatccactttgectttctctccacaggtgtccactcccaggtccaactgcaggaga
Pst 1

310 330 350
S G P G L V R P S Q T L S L T C T V S G
gcgggtccaggtcttgtgagacctagccagaccctgagcctgacctgcaccgtgtctggca

370 390 410
S T F S G Y G V N W V R Q P P G R G L E
gcaccttcagcGGCTATGGGTGTAACtgggtgagacagccacctggacgaggtcttgagg

430 450 470
W I G M I W G D G N T D Y N S A L K S R
ggattggaaTGATTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCagag

490 510 530
V T M L V D T S K N Q F S L R L S S V T
tgacaatgctggttagacaccagcaagaaccagttcagcctgagactcagcagcgtgacag

550 570 590
A A D T A V Y Y C A R E R D Y R L D Y W
ccgccgacaccgcggtctattattgtgcaagaGAGAGAGATTATAGGCTTGACTACTggg

610 630 650
G Q G S L V T V S S
gtcaaggcagcctcgtcacagttcctcaggtgagtccttacaacctctctctctattc

670 690 710
agcttaaatagattttactgcatttgttgggggggaaatgtgtgtatctgaatttcaggt

730 750 770
catgaaggactagggacaccttgggagtcagaaagggtcattgggagccgtggctgatgc

790 810 830
agacagacatcctcagctcccagacctcatggccagagatttatagggatcc
BamHI

FIG. 15. Nucleotide sequences of the (a) acceptor V_H (anti-Lys-24) and (b) V_K gene (anti-Lys-18) segments of the pMc vectors used for CDR exchange mutagenesis. The amino acid sequences are printed in single-letter code on top of the central base of a triplet. The CDR regions are given in capital letters.

50
aatcctctgaatctacatggta

110
gacacagttctctctacagtt

170
I I L F L V A T
TCATCCTCTCTTGGTAGCAAC

230
ctggacatatatatgggtgaca

290
S Q V Q L Q E
tcccaggtccaagtgcaggaga
Pst 1

350
L T C T V S G
ctgacctgcaccgtgtctggca

410
P P G R G L E
jccacctggacgaggtcttgag

470
N S A L K S R
TAATTCAGCTCTCAAATCCagag

530
L R L S S V T
cctgagactcagcagcgtgacag

590
D Y R L D Y W
AGATTATAGGCTTGACTACTggg

650
cttacaacctctctctctattc

710
atgtgtgtatctgaatttcaggt

770
jtcattgggagccgtggctgatgc

830
tgatttatagggatcc
BamHI

tor V_H (anti-Lys-24) and (b) V_K gene
CDR exchange mutagenesis. The amino
top of the central base of a triplet. The

b

10 30 50
aagctttatgaatatgcaaatcctctgaatctacatggtaaatataggtttgtctatacca
Hind 3

70 90 110
caaacagaaaaacatgagatcacagttctctctacagttactgagcacacaggacctcac

130 150 170
M G W S C I I L F L V A T A T
CATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAggtaaggggtcac

190 210 230
agtagcaggtctgaggtctggacatatatgggtgacaatgacatccactttgcctttc

250 270 290
G V H S D I Q M T Q S P S S L S A
tctccacaggtgtccactccagatccagatgacccagagcccaagcagcctgagcgcca

310 330 350
S V G D R V T I T C R A S G N I H N Y L
gcgtgggtgacagagtaccatcacctgtAGAGCCAGCGGTAAATCCACAACCTACCTGG

370 390 410
A W Y Q Q K P G K A P K L L I Y Y T T T
CTgtgtaccagcagaagccaggttaagggtccaaagctgctgatctactACACCACACCC

430 450 470
L A D G V P S R F S G S G S G T D F T F
TGGCTGACggtgtgccaaagcagattcagcggtagcggtagcggtaccgacttcaccttca

490 510 530
T I S S L Q P E D I A T Y Y C Q H F W S
ccatcagcagcctccagcagaggacatcgccacactactactgcCAGCACTTCTGGAGCA

550 570 590
T P R T F G Q G T K V E I K R
CCCCAAGGACGttcggccaagggaaggtggaaatcaaacgtgagtagaatttaaact

610
ttgcttctcagttggatcc
BamHI

FIG. 15b.

Preparation of Template and Gapped Duplex DNA

The human V genes were cloned into the *Hind*III and *Bam*HI sites of modified pMc and pMa phasmids. In order to use the mutagenic oligonucleotides as designed, the polylinkers of pMa/c had to be inverted.

For the preparation of template DNA and gapped duplex DNA we follow the protocol described in Ref. 26. The single-stranded DNA (ssDNA) is prepared from the pMc version of the vector. The double-stranded DNA (dsDNA) is prepared from the pMa version using the restriction enzymes *Xba*I and *Eco*RI to release the V gene insert from the vector.

Mutagenesis

The gdDNA is used as template for the site-specific mutagenesis to exchange the CDR regions. Usually we try to exchange all three CDRs in one round of mutagenesis. A typical experiment is as follows.

Generation of gdDNA. ssDNA (0.5 pM: 770 ng for V_L and 805 ng for V_H) is mixed with 0.1 pM dsDNA (*EcoRI/XbaI* cut) + 5 μ l 1.5 M KCl in 100 mM Tris, pH 7.5, + H₂O to 40 μ l. Incubate at 100° for 4 min and then at 65° for 10 min in water baths. Use 8 μ l for gel analysis.

Annealing and Polymerase Reaction. To 8 μ l of gdDNA, add 3 μ l of each mutagenic oligonucleotide (10 μ M/ml, kinased). Incubate at 68° for 5 min and at room temperature for 15 min. Add the following:

Fill-in buffer (10 \times): 500 mM NaCl, 100 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 10 mM DTT, 10 mM dNTPs; 4 μ l
T4 ligase (2U), 2 μ l
Klenow polymerase (2.5 U), 0.5 μ l
H₂O to 40 μ l

Incubate at room temperature for 45 min.

Selection and Screening for Mutants. Transform 5 μ l of the reaction mixture into WK6mutS²⁸ bacteria and plate aliquots on 1 \times YT²⁹ (yeast Tryptone)-Amp plates (200 μ g/ml ampicillin) and YT-chloramphenicol plates (30 μ g/ml chloramphenicol) to determine the efficiency of transformation. The remaining transformation mixture is used to inoculate 10 ml LB²⁹ (Luria-Bertani) medium (200 μ g/ml ampicillin). Bacteria are grown overnight to allow segregation of strands. Plasmid DNA is isolated²⁹ and used to transform WK6 bacteria²⁸ selecting for Amp resistance (200 μ g/ml ampicillin).

The resulting colonies are screened with the CDR3 oligonucleotides described in Fig. 4 using standard colony hybridization methods.²⁹ From the colonies hybridizing with the probe, 1.5-ml cultures are grown (YT, 200 μ g/ml ampicillin), DNA is prepared and the nucleotide sequence determined using the V_H and V_L gene sequencing oligonucleotides (Fig. 4). Five to 20% of the clones hybridizing with the CDR3 mutagenic oligonucleotide have all three CDRs placed properly.

For antibody BW431/26 the sequences of the humanized V gene versions were confirmed (Fig. 16).

²⁸ R. Zell and H.-J. Fritz, *EMBO J.* 6, 1809 (1987).

²⁹ T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

the site-specific mutagenesis to exchange all three CDRs in riment is as follows.

M: 770 ng for V_L and 805 ng for $I/XbaI$ cut) + 5 μ l 1.5 M KCl in Incubate at 100° for 4 min and e 8 μ l for gel analysis.

To 8 μ l of gdDNA, add 3 μ l of 1l, kinased). Incubate at 68° for 5 . Add the following:

mM Tris-HCl, pH 7.5, 100 mM s; 4 μ l

Transform 5 μ l of the reaction plate aliquots on 1 \times YT²⁹ (yeast cillin) and YT-chloramphenicol ermine the efficiency of transfor- mixture is used to inoculate 10 ml l ampicillin). Bacteria are grown . Plasmid DNA is isolated²⁹ and ig for Amp resistance (200 μ g/ml

with the CDR3 oligonucleotides / hybridization methods.²⁹ From 1.5-ml cultures are grown (YT, and the nucleotide sequence de- lencing oligonucleotides (Fig. 4). h the CDR3 mutagenic oligonu- erly. es of the humanized V gene ver-

BW431/26VHhum

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      10          30          50
Q V Q L Q E S G P G L V R P S Q T L S L
caggtccaactgcaggagagcggtccaggtcttgtgagacctagccagacctgagcctg

      70          90          110
T C T V S G F T I S S G Y S W H W V R Q
acctgcaccgtgtctggcTTCaccATCagcAGTGGTTATAGCTGGCACTgggtgagacag

      130          150          170
P P G R G L E W I G Y I Q Y S G I T N Y
ccacctggagcggtcttgagtggttggATACATACAGTACAGTGGTATCACAATACTAC

      190          210          230
N P S L K S R V T M L V D T S K N Q F S
AACCCCTCTCTCAAAAGTagagtgaatgctggtagacaccagcaagaaccagttcagc

      250          270          290
L R L S S V T A A D T A V Y Y C A R E D
ctgagactcagcagcgtgacagccgacacccgagcgggtctattattgtgaagaGAAGAC

      310          330          350
Y D Y H W Y F D V W G Q G S L V T V T V
TATGATTACCACTGGTACTTCGATGTCTggggtcaaggcagcctcgtcacagtcacagtc

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BW431/26VKhum

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      10          30          50
G V H S D I Q M T Q S P S S L S A S V G
ggtgtccactccgacatccagatgacccagagcccaagcagcctgagcgcagcgtgggt

      70          90          110
D R V T I T C S T S S S V S Y M H W Y Q
gacagagtgaccatcacctgtAGTACCAGCTCGAGTGAAGTTACATGCACtgggtaccag

      130          150          170
Q K P G K A P K L L I Y S T S N L A S G
cagaagccaggttaaggctccaaagctgctgatctacAGCACATCCAACCTGGCTTCTggt

      190          210          230
V P S R F S G S G S G T D F T F T I S S
gtgccaagcagattcagcggttagcggttagcggtaccgacttcaccttcaccatcagcagc

      250          270          290
L Q P E D I A T Y Y C H Q W S S Y P T F
ctccagccagaggacatcgccacctaactactgcCATCAGTGGAGTAGTTATCCACGttc

      310          330
G Q G T K V E I K R
ggccaagggaaccaaggtggaatcaaactg

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FIG. 16. Nucleotide sequences of the humanized V_H (top) and V_K (bottom) of MAb BW431/26. The sequences start with the codon for the first amino acids of the mature heavy and light chains. The human framework sequences are printed in lower-case letters while the murine CDR sequences and the murine framework sequences that have been transferred to the human V genes are printed in capital letters. The amino acids are given in the single-letter code and are printed on top of the first base of the codon.

Expression of Humanized MAb

The humanized V genes were cloned into separate eukaryotic expression vectors that contain Ig constant-region genes for the heavy and light chain, respectively (Fig. 3). The resulting V_H and V_L expression vectors were subsequently transfected into BHK cells (ATCC CCL10) using the calcium phosphate precipitation technique with methotrexate and G418 as selection markers.³⁰ We were able to isolate clones that produce up to 15 $\mu\text{g/ml}$ antibody product in serum-free roller bottle cultures without prior amplification.

Characterization of Humanized MAb

The humanized BW431/26 binds to purified CEA and to CEA on tissue sections. Competition experiments with the original mouse MAb reveal that the affinity of the reshaped antibodies, although it has not yet been determined exactly, closely resembles that of the mouse MAb, which is approximately 1×10^{-10} liter/mol.

Anti-idiotypic antibodies (anti-id) against the murine BW431/26 MAb were tested for their binding to the humanized version. Surprisingly, 17 out of 18 anti-id tested bind the humanized version as well as the mouse MAb; binding of one anti-id is less efficient but still detectable. This finding suggests a close immunological relationship between the variable domains of the mouse and the humanized BW431/26 (K. Bosslet *et al.*, unpublished data, 1991).

A comparison of the framework amino acid sequences of the donor (the murine MAb BW431/26) V_H and V_L with the acceptor V_H (human NEW) and V_L (human REI) shows a high degree of sequence similarity (70% for V_H and 68% for V_L). The homologies are clustered in the regions close to the CDRs, especially in the light chain domains. Even in positions where the amino acid sequences differ from each other the amino acid exchanges are often conservative. This may have contributed to the successful humanization of the BW431/26 antibody. The crystal structures of the REI and NEW antibodies are known and we use their respective V regions as general acceptors for the CDRs of any murine MAb. Computer modeling is used for refinement to identify potential framework amino acids in the mouse antibody that might interact with the CDRs or directly with antigen and these amino acids are transferred to the human frameworks along with the CDRs. Using the reshaping scheme described here we estimate the success rate for humanizing any particular monoclonal antibody to be approximately 80%.

³⁰ M. Wirth, J. Bode, G. Zettlmeissl, and H. Hauser, *Gene* 73, 419 (1988).

to separate eukaryotic expression genes for the heavy and light V_H and V_L expression vectors (ATCC CCL10) using the cells with methotrexate and G418 as selection clones that produce up to 10⁸ cells in roller bottle cultures without

purified CEA and to CEA on the original mouse MAb antibodies, although it has not yet been shown that of the mouse MAb, which

is not the murine BW431/26 MAb reshaped version. Surprisingly, 17 out of 20 as well as the mouse MAb; but still detectable. This finding is in agreement between the variable domains of the murine BW431/26 (K. Bosslet *et al.*, unpublished)

no acid sequences of the donor V_L with the acceptor V_H (human V_H) degree of sequence similarity. The regions of sequence similarity are clustered in the regions of the chain domains. Even in positions far from each other the amino acid sequence may have contributed to the structure of the antibody. The crystal structures of the donor and we use their respective V sequences of any murine MAb. Computer analysis of potential framework amino acid interactions with the CDRs or directly transferred to the human framework reshaping scheme described here we can see any particular monoclonal anti-

Gene 73, 419 (1988).

Queen *et al.*³¹ have reshaped an antibody by selecting human acceptor V domains from the Kabat database to match the framework sequences of the murine donor V domains as closely as possible. This alternative reshaping strategy raises the possibility that any human antibody framework can be used as acceptor for the murine CDRs. Humanization of murine MABs is a technique that is now widely applied to MABs of potential use in diagnosis and therapy in humans. Whether one of the reshaping schemes will offer any particular advantage in successful humanization of MABs will become clearer as the number of reshaped antibodies increases.

Acknowledgments

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³¹ C. Queen, W. P. Schneider, H. E. Selick, P. W. Payne, N. F. Landolfi, J. F. Duncan, N. M. Avdalovitch, M. Levitt, R. P. Junghans, and T. A. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* 86, 10029 (1989).

[6] Molecular Modeling of Antibody Combining Sites

By ANDREW C. R. MARTIN, JANET C. CHEETHAM, and
ANTHONY R. REES

Introduction

Both the variable and constant domains of an antibody Fab consist of two twisted antiparallel β sheets which form a β -sandwich structure. The constant regions have three- and four-stranded β sheets arranged in a Greek key-like motif,¹ while variable regions have a further two short β strands producing a five-stranded β sheet.

The two β sheets of the variable domain are inclined at 30° to one another,² with a conserved disulfide bridge in each domain linking the two β sheets. Lesk and Chothia³ have shown the relative orientation of the two

¹ J. S. Richardson, *Adv. Protein Chem.* 34, 168 (1981).

² C. Chothia and J. Janin, *Proc. Natl. Acad. Sci. U.S.A.* 78, 4146 (1981).

³ A. M. Lesk and C. Chothia, *J. Mol. Biol.* 160, 325 (1982).